IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Nicholas W. Warne		
Serial No.	:	08/230,982	Examiner:	C. Sayala
Filed	:	April 21, 1994	Art Unit:	1815
For	:	FORMULATIONS FOR IL-11		

August 1, 1995

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

DECLARATION OF DR. NICHOLAS W. WARNE AND ATTACHED *CURRICULUM VITAE*

I, Nicholas W. Warne, declare and state:

1. I am the named inventor in the above-identified application.

2. I hold a Bachelor of Science degree in Biochemistry from the University of Rochester (B.S. with Distinction in Research, 1984) and a Doctorate in Chemistry (1990) from Purdue University.

3. I am a member of the Protein Society, and the American Association of Pharmaceutical Scientists.

4. I have authored a number of peer-reviewed publications in the field of protein structural and physicochemical analyses, and have given lectures on the topics of protein stability and formulation development.

CERTIFICATE OF MAILING

Date of Deposit 8-1-95

I hereby certify that the above-specified paper(s) and/or fee(s)is being deposited with the U.S. Postal Service under 37 CFR §1.10, in an envelope addressed to Honorable Commissioner of Patents and Trademarks, Washington, D.C., 20231, on the date of deposit as indicated above.

Exhibit 6
USSN 08/230,982
GI 5218

5. In 1990, I joined Genetics Institute employed as a Staff Scientist I in the Process Biochemistry Group to develop parenteral dosage forms of protein biopharmaceuticals. I have developed dosage forms for the growth factor FGF4, antibody 3F8, and the cytokine IL-12, as well as for IL-11.

6. My research focus has been primarily in the area of stabilizing proteins against effects of manufacturing and storage. I am interested in the development and characterization of protein dosage forms.

7. Over the years, I have become very familiar with the research undertaken by other scientists in the protein stability and formulation development field and with their publications.

8. This declaration addresses the obviousness issue raised by the Examiner in the Office Actions dated January 9, and May 15, 1995 and provides factual/evidentiary support for Applicant's assertion that there was no "reasonable expectation of success" of a stabilizing IL-11 formulation.

9. I have read all of the Examiner's Office Actions and the references cited as well as Applicant's Response filed February 9, 1995 and the Response being filed with this Declaration. I have also read all of the Exhibits previously filed with Applicant's Response and I am also familiar with the Exhibits being supplied with this Response.

10. It is my understanding that at issue is whether the claimed IL-11 compositions are unobvious under 35 USC §103 over two primary references (Wang and Manning) in view of six secondary references (Paul, Hamblin, Kwan, Kato, Singh, and Patel).

11. It is my understanding that the Examiner believes that it would have been obvious to combine the teaching of Wang (glycine as a stabilizer) and Manning (degradation mechanisms) in combination with Paul (IL-11); Hamblin (cytokines); Patel (interferon-α-2β/methionine, histidine; GM-CSF/methionine, histidine; IL-4/methionine, histidine); Singh (tumor necrosis factor/HSA, dextran, PEG, polysorbate-80, polyvinylpyrrolidone, sucrose, lactose, trehalose; tumor necrosis factor/citrate, phosphate, citrate-phosphate; tumor necrosis factor/glycine, mannitol); Kwan (α-2-interferon/glycine, alanine; α-1-

interferon/glycine, alanine); and Kato (γ-interferon/arginine, histidine, lysine, hydroxylysine, ornithine, glutamine, γ-aminobutyric acid, ε-aminocaproïc acid).

12. I disagree. The factual basis for my opinion is set forth below.

FUNCTIONAL SIMILARITIES DO NOT PREDICT STRUCTURAL HOMOLOGIES

13. Many functionally similar proteins do not share structural similarities. Stabilization effects observed with one protein cannot predictably be extended to another protein. Proteins are classified structurally and/or functionally. Classifications based on **function** teach nothing about a protein's **structural** stability requirements. Classifications based on **structure** only sometimes provide hints about what might be tried.

14. IL-11 is **structurally** (chemically) distinguishable from all of the proteins/peptides cited in the references. That IL-11 has been termed an "interleukin" and a "cytokine" is a **functional**, not a **structural** distinction. To reiterate, **structurally**, interleukins are a diverse group of proteins, and structural information concerning any one interleukin does not predict structure of another interleukin.

15. "Interleukin" is a **functional** term. It is applied to those proteins which are secreted by **leukocytes** (white blood cells) and which act as growth factors or regulatory mediators upon other **leukocytes**, *i.e.*, "inter" (among) "leu" (white). The interleukin nomenclature system is based on **functional** not **structural** similarities. By the **functional** definition of interleukin, GM-CSF (granulocyte macrophage-colony stimulating factor) and LIF (leukocyte inhibiting factor) are interleukins; whereas EPO (erythropoietin, made by **kidney** cells) and IL-11 (interleukin-11, made by **fibroblasts**) are not **functionally** interleukins. The term "cytokine" is also a **functional** not **structural** term, meaning a factor secreted by an unspecified cell source and acting upon an unspecified cell target.

16. Structural similarities among the interleukins are at the limit of detection by computer analysis. Interleukins range in molecular weight more than seven fold. For example, IL-8 is a small (~10 kD) protein that is actually a member of the inflammatory cytokine gene family, while IL-12 is a 70 kD heterodimer. It is not possible to make any generalizations about how these molecules will behave in any given solution, *i.e.*, in a formulation. Indeed, IL-8 shares no homology with other interleukins. Certain other cytokines, though they are not designated as "interleukins", actually share greater structural similarities with other cytokines that are designated as interleukins. For example, IL-3 (designated an interleukin) is more closely related to GM-CSF (not designated an interleukin) than it is to proteins that are interleukins.

17. Function does not predict structure. Zav'yalov, *et al.*, Biochim. et Biophys. Acta. *1041*:178 (1990) (Exhibit 1) presented a theoretical conformational analysis of a family of α-helical immunocytokines and pointed out that certain functionally dissimilar molecules have some primary structural similarity, whereas certain functionally similar molecules do not share structural similarity. The 1992 publication, Manvalan, *et al.*, J. Protein Chem. *11*:321 (1992) (Exhibit 2) concluded (page 321, second column):

> Sequence homologies among cytokine ligands, unlike their receptors, are not apparent, except that IL-6 shows limited homology with G-CSF and cMGF.

IL-6 is an interleukin; G-CSF is a granulocyte-colony stimulating factor; and cMGF is a growth factor; *i.e.*, the molecules are functionally unrelated, yet they share some structural similarity. See also, Bruce, *et al.*, Progress in Growth Factor Res. *4*:157 (1992). (Exhibit 3). Figure 2 identifies four regions having "singlet" common amino acids; however, not a single amino acid is always present in the array of molecules displayed. See also, Minasian, *et al.*, Protein Seq. Data Anal. *5*:57 (1992) (Exhibit 4), which provides a summary of select cytokine structures. Fig. 1, an alignment of the protein sequences of 18 different cytokines, clearly demonstrates that there are no conserved sequences. Thus, there is no structural homology among this functional "class" of proteins.

18. Strober, *et al.*, Ped. Res. *24*:549 (1988) (Exhibit 5) note that ". . . the various designations have little to do with the origin or the function of the individual molecules." (p.549, col. 1). Indeed, interleukins are produced by leukocytes, while IL-11 is produced by fibroblasts. Thus, the cell source of the proteins are distinct.

19. Contrary to the Examiner's assertion that IL-11 is "analogous" to various cytokines and interleukins, in fact, IL-11 is not structurally analogous nor homologous to the proteins of the references. In Manvalan, *et al.* (Exhibit 2), the authors, compared the protein sequence of IL-11 with those of the cytokine superfamily, and concluded that structurally IL-11 is not a cytokine (p.329, col.1). Thus, formulations effective with the cytokines of the references such as GM-CSF, α-2-interferon, β-1-interferon, and γ-interferon are not predictive of whether such would be successful with IL-11.

20. A structural comparison of the amino acid sequences of IL-11 with various cytokines and interleukins (Minasian *et al.*, fig. 1) (Exhibit 4), demonstrates that IL-11 is quite different from the other proteins examined. **IL-11 shows no sequence similarity to any other protein listed. IL-11 is not homologous to any other known protein sequence in any sequence database.** Table 3 lists the predicted 4-α-helical bundle structures predicted for various cytokines and interleukins. IL-11 does not have many of the common features found in the listed proteins. IL-11 has no cysteines, no disulfide bonds, and no N-linked glycosylation sites. The lack of these features dramatically decreases IL-11's solubility and stability characteristics.

21. Disulfide bonds typically add much needed structural stability to proteins, increasing the thermal denaturation temperature and decreasing the potential for chemical denaturation. IL-11 has no disulfide bonds.

22. Glycosylation of a protein greatly enhances its solubility. IL-11 has no glycosylation. In contrast, IL-4 has three disulfide bonds and three N-linked glycosylation sites to which carbohydrate is attached.

23. The lack of disulfide bonds and lack of N-linked glycosylation of IL-11 make IL-11 an unusual and distinctive protein.

24. Most proteins are 5-10% leucine. IL-11 has a distinctively high leucine and arginine content. IL-11 is 23% leucine (making the protein core extremely hydrophobic), and 10% arginine (making the protein extremely basic, pI ~ 12).

25. This unusually high leucine content of 23% makes IL-11 far more susceptible to precipitation and/or aggregation than proteins having only 5-10% leucine. IL-11 actually precipitates upon thermal denaturation. In contrast, a cytokine like M-CSF remains soluble upon thermal denaturation.

26. The unusually high arginine content and resultant extremely basic pI of IL-11 make it impossible to generalize solubility characteristics based on proteins that are not similarly basic. No one before me was confronted with the problem of stabilizing such an "insoluble" and "basic" protein.

STABILIZATION IS UNPREDICTABLE

27. As one skilled in the art, I assert that stabilization of any protein is unpredictable; in particular, the stabilization of such a distinctive protein as IL-11 is wholly unpredictable. Nothing in the prior art instructs how to stabilize such a basic molecule. At pages 6-7, the Examiner states that there is no requirement that:

> . . . stabilization methods should be based on homology.

28. As one skilled in the art, I assert that stabilization methods do in fact take into account the structural features and needs of the molecule to be stabilized. Nothing in Wang nor in Manning teaches the specific problems to overcome in stabilizing IL-11, *i.e.*, the lack of IL-11's solubility in combination with an extremely hydrophobic core and a propensity to aggregate. I discovered the problem, and, by my invention, I provided the solution.

29. I have found that some of the chemical instability of IL-11 is a result of hydrolysis between Asp^{133} and Pro^{134}. Also, deamidation of Asn^{49} to Asp^{49} is detected. In addition, oxidation of Met^{58} is observed.

All of these chemical reactions are evidence of IL-11 protein chemical instability. IL-11 is also subject to certain physical instabilities including a dimerization process (which is actually a shift in equilibrium between the monomeric and dimeric forms of IL-11), as well as aggregate formation.

30. According to my invention, the addition of glycine acts to prevent aggregation of IL-11 and protects IL-11 from the harmful effects of shearing. This in turn increases the ability to handle the protein and provides enhanced shelf-life for IL-11 products. Addition of an appropriate buffering agent slows the rate of hydrolysis, deamidation, and oxidation.

31. The results set forth in Table I (Example 1 of specification) clearly demonstrate the unpredictable results obtained with a broad range of IL-11 solutions. The percent recovery of IL-11 varies from a low of 47% to a high of 98%.

Table I
Excipient Effects on Shearing of rhIL-11

Base Buffer	Additive	Structural Formulae	pH	Percent Recovery
50 mM sodium phosphate	150 mM sodium chloride	NaCl	6.0	74
	1 M sodium chloride	NaCl	6.0	65
10 mM Histidine	1 M sodium chloride	NaCl	7.5	63
	20 mM calcium chloride	$CaCl_2$	7.5	89
	20 mM magnesium chloride	$MgCl_2$	7.5	89
	0.2 M glycine	NH_2CH_3	7.5	97
	0.5 M sodium chloride	NaCl	7.0	71
	0.2 M ethyl glycine	$NH_2CH_2CH_2CH_3$	7.0	98
	0.2 M β-amino-n-propionic acid	$NH_2CH_2CH_2COOH$	7.0	77
	0.2 M γ-amino-n-butyric acid	$NH_2CH_2(CH_2)_2COOH$	7.0	47
	0.2 M δ-amino-n-valeric acid	$NH_2CH_2(CH_2)_3COOH$	7.0	63
	0.2 M ϵ-amino-n-caproic acid	$NH_2CH_2(CH_2)_4COOH$	7.0	84

32. Surprizingly, the greatest shear-protecting effects are observed with glycine (1-carbon), ethyl glycine (3-carbon), magnesium chloride (no carbon), calcium chloride (no carbon), and ϵ-amino-n-caproic acid (6-carbon). These "protectants" are not structurally related to each other. Surprizingly, the amino carboxylic acids which differ only in carbon length (ranging from 3 to 6 carbons in length), actually have

the greatest variability, ranging from 47 to 84%. Surprizingly, the 3-carbon and 6-carbon lengths are better than the 4-carbon and 5-carbon lengths. However, the 1-carbon glycine and 3-carbon glycine, surprizingly, behave similarly to each other.

33. Also surprizing is that calcium chloride alone or magnesium chloride alone are better than sodium chloride alone, sodium chloride with histidine, and any of the amino carboxylic acids.

34. Surprizingly, in the process of simply concentrating an IL-11 solution, utilizing glycine increases IL-11 recoveries from a range of 85-90% to a range of 98-100%. (Example 2).

35. Surprizingly, the addition of glycine increases the temperature to which IL-11 will remain soluble. (Example 3).

36. Surprizingly, when IL-11 is stored up to 12 months, the addition of more glycine aids in increasing the shelf-life stability of IL-11 in the liquid state, at elevated temperatures. Further, the absence of glycine altogether leads to a dramatic loss of rhIL-11 at elevated temperatures. (Example 4).

37. rhIL-11 is prepared, at 5.0 mg/mL in two formulations: 10 mM sodium phosphate, 300 mM glycine, pH 7.0 and 10 mM sodium phosphate, 150 mM glycine, pH 7.0. One mL samples are prepared in 2-mL molded vials (Kimble), stoppered and crimped, and incubated at six temperatures for up to 12 months (-80°C, -20°C, 2-8°C, 30°C, 40°C, 50°C). Protein recoveries are determined using a reversed-phase HPLC method and the results are shown in Table III.

Table III

Effect of Glycine Concentration on Percent rhIL-11 Recoveries at Different Temperatures

INCUBATION TEMPERATURE	10 mM sodium phosphate 300 mM glycine, pH 7.0	10 mM sodium phosphate 150 mM glycine, pH 7.0
-80° C at 12 months	100	100
-20° C at 12 months	96.9	97.2
2-8° C at 12 months	98.3	100
30° C at 12 months	91.5	71.7
40° C at 6 months	72.4	63.2
50° C at 2 months	72.9	75.5

38. Another set of samples is prepared in a formulation of 10 mM sodium phosphate, 300 mM glycine, pH 7.0. These samples are liquid and stored at 2-8°C for up to 24 months. Surprizingly, the samples retain IL-11 activity.

39. Another set of samples is prepared in a formulation of 20 mM L-histidine, 300 mM glycine, pH 7.0. These samples are lyophilized and stored at 2-8°C for up to 24 months. Surprizingly, the samples retain IL-11 activity.

THE ART TEACHES AWAY FROM USING GLYCINE

40. Glycine as a potential protein stabilizer is disclosed in Wang. Wang teaches the unsuitability of glycine (at page S12, column 2, third paragraph):

> Likewise, Takagi (1980), in order to formulate an intravenous immune globulin G preparation that did not increase in anticomplement activity when stored as a solution, added arginine or lysine as stabilizers; ornithine, aspartic acid, glutamic acid, alanine, and **glycine had no stabilizing effects.** (Emphasis supplied)

Wang teaches (Table III, page S14) that for more than 80% of the formulations listed glycine alone is insufficient, *i.e.*, other ingredients are required to achieve stability such as EDTA, gelatin, albumin, sodium borate, mannitol, saccharose, citrate, and/or human serum albumin (HSA). Given the myriad specific formulations for specific proteins that Wang discloses it is clear that whether a particular formulation will provide a stable environment for the protein of interest is unpredictable. The reference says nothing about how to stabilize IL-11 *per se*.

41. Manning provides no guidance and merely discloses the problems to be overcome: deamidation, oxidation proteolysis, incorrect disulfide formation, racemization, β-elimination and protein instabilities (such as denaturation, surface adsorption and precipitation). Manning provides no instruction on the problems in stabilizing IL-11 *per se*.

THE ART TEACHES AWAY FROM USING GLYCINE IN COMBINATION TO STABILIZE IL-11

42. The six secondary references neither disclose nor suggest the use of buffering agents in combination with glycine to stabilize IL-11. Paul discloses IL-11. Hamblin discloses cytokines. A summary of the specific components disclosed in the other four secondary references is set forth below.

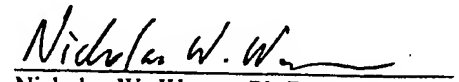
Reference	Protein	Formulation Components
Patel	interferon-α-2β	methionine, histidine
	GM-CSF	methionine, histidine
	IL-4	methionine, histidine
Singh	tumor necrosis factor	HSA, dextran, PEG, polysorbate-80, polyvinylpyrrolidone, sucrose, lactose, trehalose
	tumor necrosis factor	citrate, phosphate, citrate-phosphate
	tumor necrosis factor	glycine, mannitol
Kwan	α-2-interferon	glycine, alanine
	α-1-interferon	glycine, alanine
Kato	γ-interferon	arginine, histidine, lysine, hydroxylysine, ornithine, glutamine, γ-aminobutyric acid, ε-aminocaproic acid

43. Patel teaches away from the use of histidine. Patel teaches, in Example 3, an interleukin-4 composition and teaches (figure 4) that histidine actually decreases the stability of IL-4 relative to the control. Histidine is, therefore, a destabilizer. There is no disclosure or suggestion that histidine would be effective in stabilizing IL-11. Indeed, the only interleukins disclosed (column 3, lines 24 *et seq.*) are IL-1, IL-2, IL-3, IL-4 and IL-10. There is no disclosure of IL-11.

44. Singh and Kwan disclose phosphate buffer to stabilize tumor necrosis factor and alpha-interferon. They do not disclose nor suggest that the buffering agent could be used in combination with glycine specifically to stabilize IL-11. Kato discloses stabilization of interferon with histidine and buffer salts, but there is no disclosure or suggestion to use glycine with a buffering agent to stabilize IL-11.

45. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

August 1, 1995.



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Professional Societies:

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Publications:

Photodynamic Inactivation of Selected Intracellular Enzymes by Hematoporphryn Derivitive and Their Relationship to Tumor Cell Viability *in vitro*, R.Hilf, N.W.Warne, D.B.Smail and S.L.Gibson, (1984) Cancer Letters 24, 165-172

All Fifteen Possible Arrangements of Three Disulfide Bridges are Known, N.W.Warne and M.Laskowski,Jr., (1990) BBRC 172, 1364-1370

Acid-Catalyzed Peptide Bond Hydrolysis of Recombinant Human Interleukin 11, R.A.Kenley and N.W.Warne, (1984) Pharm. Res. 11, 72-76

Abstracts of Papers Presented:

Chemical Synthesis and Characterization of Turkey Ovomucoid Third Domain, A Serine Proteinase Inhibitor, N.W.Warne, M.A.Qasim, T.L.Bigler, M.Weiczorek and M.Laskowski, Jr., Third Symposium of the Protein Society, Seattle, WA, July 29-August 2, 1989

All Fifteen Possible Arrangements of Three Disulfide Bridges are Known, N.W.Warne and M.Laskowski, Jr., Fourth Symposium of the Protein Society, San Diego, CA, August 1990

Expression of Turkey Ovomucoid Third Domain and of Several Variants with Altered P1 Residues, W.Lu, T.Komiyama, N.Warne, M.Laskowski,Jr., Y.Chiang, I.Rothberg, K.Ryan and S.Anderson, Sixth Symposium of the Protein Society, San Diego, CA, July 1992

Structural Stability of Protein Biopharmaceuticals, lecture presented at the University of Kentucky College of Pharmacy, April 13, 1992

Chromatographic Characterization of a Recombinant Human Protein, I.Mazsaroff, N.Warne, M.Czupryn, J.Amari, M.Fleming and J.Wright, Twelfth International Symposium on HPLC of Proteins, Peptides and Polynucleotides, November 29-December 2, 1992

Characterization of a Recombinant Human Protein Using High Performance Capillary Electrophoresis, K.Ganzler, I.Mazsaroff, M.Czupryn, N.Warne and J.Wright, Fifth International Symposium on HPCE, Orlando, January 25-29, 1993

Use of Biophysical Techniques as Stability Indicating Assays, K.W.Yim, N.W.Warne, J.Steckert and R.A.Kenley, Eighth Annual Symposium of the AAPS, Orlando, November 14-18, 1993

Peracetic Acid Oxidation of rhIL-11, N.W.Warne, T.R.Crowley, R.A.Kenley, K.W.Yim and G.Amphlett, Eighth Annual Symposium of the AAPS, Orlando, November 14-18, 1993

The Stability of rhIL-11 as a Function of pH, Time and Temperature, R.Ingram and N.Warne, Ninth Annual Symposum of the AAPS, San Diego, November 6-10, 1994

Histidine-Patch Thioredoxins: Mutant Forms of Thioredoxin with Metal Chelating Affinity Which Provide for Convenient Purifications of Thioredoxin Fusion Proteins, Z.Lu, L.DiBlasio-Smith, K.Grant, N.Warne, E.LaVallie, L.Racie, M.Follettie, M.Williamson and J.McCoy, Ninth Symposium of the Protein Society, Boston, July 8-12, 1995